IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION OF ACCURACY OF TRANSLATION IN LIEU OF SWORN TRANSLATION (37 C.F.R. 1.55 6 1.68)

The undersigned translator, having an office at E-1 Bldg., 3-12, Nihonbashi 3-chome, Chuo-ku, Tokyo, Japan certifies and declares that:

(1) I am fully conversant both with the Japanese and English languages.

(2)(A) I have translated into English Japanese Patent Application Number 2004-019251, filed on January 28, 2004. A copy of said English translation is attached hereto.

(2)(B) I have carefully compared the attached English language translation of Japanese Patent application Number filed with the original Japanese-language patent application.

(3) The translation is, to the best of my knowledge, and belief, an accurate translation from the original into the English language.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the matter with which this translation is used.

Date: February 4, 2010

Hiroya YANO

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this office.

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Applicant: Mitsukan Group Corporation

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Hiroshi Ogawa
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[Name of document] Claims

[Claim1] A polypeptide NAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with a polypeptide NBS shown below in (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 2] A polypeptide NAS according to claim 1, which is glycosylated with an N-linked sugar chain comprising mannose/N-acetylglucosamine/fucose/xylose at a ratio of 3/2/1/1.

[Claim 3] DNA of a gene encoding a polypeptide NAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with a polypeptide NBS shown below in (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 4] DNA of a gene according to claim 3, which is a DNA shown below in (A) or (B):

(A) DNA containing the nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing;

(B) DNA hybridizing with the DNA of a nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or with the DNA of a nucleotide sequence capable of functioning as a probe prepared from at least a part of the nucleotide sequence under stringent conditions and encoding a polypeptide capable of forming the neoculin dimer having a taste-modifying activity

together with a polypeptide NBS shown below in (a) or (b):
(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;
(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 5] A polypeptide PNAS shown below in (A) or (B):
(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing;
(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing, which polypeptide grows to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with a polypeptide NBS shown below in (a) or (b):
(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;
(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 6] A polypeptide PNAS according to claim 5, which is glycosylated with an N-linked sugar chain comprising mannose/N-acetylglucosamine/fucose/xylose at a ratio of 3/2/1/1.

[Claim 7] DNA of a gene encoding a polypeptide PNAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing, which polypeptide grows to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with a polypeptide NBS shown below in (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 8] DNA of a gene according to claim 7, which is a DNA shown below in (A) or (B):

(A) DNA containing a nucleotide sequence comprising the

nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO. 1 in the sequence listing;

(B) DNA hybridizing with the DNA of the nucleotide sequence comprising the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or with the DNA of a nucleotide sequence capable of functioning as a probe prepared from at least a part of the nucleotide sequence under stringent conditions and encoding a polypeptide growing to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with a polypeptide NBS shown below in (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 9] A dimeric protein neoculin comprising a polypeptide NAS according to claim 1 or 2 and a polypeptide NBS shown below in (a) or (b) and having a taste-modifying activity:

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) a polypeptide comprising an amino acid sequence with the

substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[Claim 10] A taste-modifying composition containing the dimeric protein neoculin according to claim 9 as the active ingredient.

[Name of document] Specification

[Title of the invention] Novel taste-modifying polypeptide NAS, DNA thereof and use thereof

[Technical field]

[0001] The present invention relates to a novel taste-modifying polypeptide NAS, the DNA thereof and the use thereof. More specifically, the invention relates to the polypeptide NAS, the gene thereof, the dimeric protein neoculin containing the polypeptide and having a taste-modifying activity, and a taste-modifying composition containing neoculin.

[Background art]

[0002] Curculigo latifolia is a plant spontaneously growing in west Malaysia and a southern part of Thailand, which is classified into Liliaceae. It is said that the curculin isoform (referred to as curculin hereinafter) contained in the plant is useful as a taste-modifying substance giving sweet taste when the isoform is eaten before drinking water or eating a sour substance.

[0003] Conventionally known subunits constituting curculin include for example curculin A and curculin B.

The entire amino acid sequence of curculin A has been determined (see for example patent literature 1). The entire amino acid sequence of curculin B and the nucleotide sequence thereof are disclosed. It is verified that curculin B is

different in the amino acid composition from curculin A in terms of several amino acids (see for example patent literature 2).

It has been understood that these subunits both constitute curculin in a form of homodimer and have a taste-modifying function. However, the taste-modifying functions thereof are not sufficient enough to be added into foods.

In such circumstances, it has been desired to screen for a substance with higher practical applicability and a better taste-modifying function.

[0004] [Patent literature 1] JP-A-Hei 3-190899

[Patent literature 2] JP-A-Hei 6-189771

[Disclosure of the invention]

[Problems that the invention is to solve]

[0005] As described above, it is an object of the invention to find a substance with a better taste-modifying function and determine the structure of the taste-modifying substance, as well as to elucidate the structure thereof at a gene level and determine the primary structure of the substance and obtain the gene encoding the substance. Additionally, it is an object of the invention to provide a novel taste-modifying composition characteristically containing the taste-modifying substance.

[Means for solving the problems]

[0006]The inventors have made investigations so as to solve

the problems. Thus, the inventors have successfully found a novel dimeric protein with a heterodimer structure different from that of curculin, in a fruit extract from Curculigo latifolia, which has an excellent taste-modifying activity. Thus, the inventors have designated the protein neoculin.

The inventors have found that neoculin greatly reduces the sourness, bitterness or astringency of foods and drinks and additionally that neoculin has an activity to enhance the taste of foods and drinks, namely a taste-modifying activity. The inventors have found that neoculin has a far better taste-modifying action than that of curculin and is highly practically applicable.

[0007] In other words, the inventors have focused their attention to the fact that neoculin is a heterodimer of a novel subunit NAS (neoculin acidic subunit) never having been known and a subunit NBS (neoculin basic subunit) such as curculin A or B having been known as subunits of curculin, in the course of purifying neoculin and determining the structure thereof.

The inventors have carried out the structural analysis of the novel subunit NAS. Compared with the homology between the known curculin A and B constituting NBS, a novel polypeptide with a lower homology has been obtained.

The inventors also have analyzed the DNA of the gene encoding the polypeptide NAS to elucidate neoculin at the gene level. The inventors have found, besides the nucleotide

sequence of the mature form of the protein NAS, the nucleotide sequence of a precursor protein (PNAS) containing a signal peptide and an extension peptide.

Furthermore, the inventors have verified that neoculin has a taste-modifying activity when added actually to foods and drinks.

The invention has been based on the findings described above.

[0008] The invention according to claim 1 is a polypeptide NAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with a polypeptide NBS shown below in (a) or (b):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

The invention according to claim 2 is the polypeptide NAS according to claim 1, which is glycosylated with an N-linked sugar chain comprising mannose/N-acetylglucosamine/fucose/ xylose at a ratio of 3/2/1/1.

[0009] The invention according to claim 3 is DNA of a gene encoding the polypeptide NAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

The invention according to claim 4 is the DNA of the gene according to claim 3, which is a DNA shown below in (A) or (B):

(A) DNA containing the nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing;

(B) DNA hybridizing with the DNA of the nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or with the DNA of a nucleotide sequence capable of functioning as a probe prepared from at least a part of the nucleotide sequence under stringent conditions and encoding a polypeptide capable of

forming the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

[0010] The invention according to claim 5 is a polypeptide PNAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing, which polypeptide grows to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

The invention according to claim 6 is the polypeptide PNAS according to claim 5, which is glycosylated with an N-linked sugar chain comprising mannose/N-acetylglucosamine/fucose/xylose at a ratio of 3/2/1/1.

[0011] The invention according to claim 7 is DNA of a gene encoding the polypeptide PNAS shown below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing, which polypeptide grows

to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

The invention according to claim 8 is the DNA of the gene according to claim 7, which is the DNA shown below in (A) or (B):

(A) DNA containing a nucleotide sequence comprising the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO. 1 in the sequence listing;

(B) DNA hybridizing with the DNA of the nucleotide sequence comprising the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or with the DNA of a nucleotide sequence capable of functioning as a probe prepared from at least a part of the nucleotide sequence under stringent conditions and encoding a polypeptide growing to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

[0012] The invention according to claim 9 is a dimeric protein neoculin comprising the polypeptide NAS according to claim 1 or 2 and the polypeptide NBS shown above in (a) or (b) and having a taste-modifying activity.

The invention according to claim 10 is a taste-modifying composition containing the dimeric protein neoculin according to claim 9 as the active ingredient.

[Effect of the invention]

[0013] In accordance with the invention, a novel dimeric protein neoculin with an excellent taste-modifying activity and in a heterodimer structure different from that of curculin is provided. Using the protein, a novel taste-modifying composition practically applicable to foods and the like is provided.

In accordance with the invention, further, the amino acid sequence of a subunit constituting the protein is provided. Thus, the protein can be provided by an appropriate synthetic method following the amino acid sequence. In accordance with the invention, the DNA of the gene encoding the protein is provided. Selecting appropriate hosts and using genetic engineering technology, the protein can be provided.

[Best mode for carrying out the invention]

[0014] (1) Dimeric protein neoculin of the invention

The neoculin of the invention is a dimeric protein comprising the polypeptide NAS and the polypeptide NBS shown below in (a) or (b) and having a taste-modifying activity.

(a) A polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing.

(b) A polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can

be a subunit constituting curculin.

As described below in (2), the polypeptide NAS (neoculin acidic subunit) is a protein first reported by the inventors. Additionally, the polypeptide NBS (neoculin basic subunit) means a polypeptide shown in (a) or (b). As described below in (2), specifically, the polypeptide NBS means known curculin subunits such as curculin A and curculin B.

Both the polypeptides form a stable heterodimer via binding with a sugar chain.

[0015] The neoculin of the invention can be obtained, for example, from Curculigo latifolia as a plant belonging to Liliaceae by an appropriate combination of known isolation and purification methods.

As described in Example 1(1), for example, a freeze-dried fruit of Curculigo latifolia is homogenized to generate a powder, which is extracted in a large volume of pure water. Via centrifugation, the resulting supernatant is discarded, to remove unnecessary materials. The remaining precipitate is extracted in an aqueous acidic solution of pH 2.0 or less, to obtain neoculin in the extract solution. Subsequently, the extract solution is neutralized, concentrated, desalted and dried by general processing methods (preferably, a method without heating), to obtain the protein neoculin sufficient enough for practical application.

The polypeptide NAS described below in (2) is

artificially prepared, and then, the polypeptide NBS obtained either by extraction and purification from neoculin or known curculin or by artificial synthesis is bound to the resulting NAS, to obtain neoculin.

[0016] The neoculin of the invention has a taste-modifying activity. Herein, the phrase taste-modifying activity means an activity prominently reducing sourness, bitterness or astringency as well as enhancing the taste of foods or drinks. Specifically, the activity means an activity suppressing the bitterness of bitter foods or drinks, an activity suppressing the astringency of foods or drinks with astringent taste, an activity giving sweetness to foods or drinks, an activity giving sweetness to sour foods or drinks, and an activity suppressing the sourness of sour foods or drinks.

[0017] (2) Polypeptide NAS of the invention

The polypeptide NAS of the invention is a polypeptide shown below in (A) or (B).

(A) A polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing.

(B) A polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown below in (a) or (b).

(a) A polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing;

(b) A polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin.

[0018] (A) a polypeptide comprising the amino acid sequence shown in SEQ ID NO. 2 in the sequence listing is a subunit first identified by the inventors as one of subunits constituting the neoculin dimer having a taste-modifying activity, together with the polypeptide NBS.

As described above, the polypeptide NBS (neoculin basic subunit) means (a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing, or (b) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several, preferably one to 5 amino acids in the amino acid sequence shown in SEQ ID NO.6 in the sequence listing, which polypeptide can be a subunit constituting curculin. Such polypeptide NBS specifically includes the polypeptide comprising an amino acid sequence shown in SEQ ID NO.6 in the sequence listing (curculin B), a polypeptide obtained by substituting tryptophan-73 in the amino acid sequence with asparagine (referred to as curculin B'), and a polypeptide

obtained by substituting lysine-28, tryptophan-73, tryptophan-78 and asparagine-81 with asparagine, asparagine, cysteine and alanine, respectively (curculin A).

[0019] Thus, the polypeptide NAS of the invention may be (A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing and may be a polypeptide substantially identical to the polypeptide (A), namely (B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several, preferably one to 5 amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing and being capable of forming the neoculin dimer having a taste-modifying activity together with the polypeptide NBS.

[0020] The polypeptide NAS is preferably glycosylated with a sugar chain, particularly preferably an N-linked sugar chain because the polypeptide NAS can get an increased binding to the polypeptide NBS so that the polypeptide NAS can form neoculin with a higher stability. Herein, the N-linked sugar chain means the general name of a sugar chain structure extending from N-acetylglucosamine, as the start point, bound to the asparagine residue existing in the primary structure of protein.

Among the N-linked sugar chains, an N-linked sugar chain comprising mannose/N-acetylglucosamine/fucose/xylose at a ratio of 3/2/1/1 is preferable. Specifically, the N-linked

sugar chain is preferably a sugar chain in the structure shown in Fig. 8. Furthermore, a part of the structure shown in Fig. 8 may have addition, deletion, substitution or modification.

From the standpoint of the binding feature of such N-linked sugar chain, the binding site of the N-linked sugar chain in the polypeptide NAS may possibly be asparagine-81 in the amino acid sequence shown in SEQ ID NO. 2 in the sequence listing.

[0021] The polypeptide NAS of the invention is a subunit forming neoculin and can be obtained from a fruit of Curculigo latifolia containing neoculin by a combination of known isolation and purification methods. As described in Example 1 (2) and (3), neoculin obtained by the method described in Example 1 (1) is purified by an appropriate combination of known ion exchange chromatographic methods. As described in Example 2, then, the purified product is applied to a cation exchange column by general methods, so that the polypeptide NBS with an isoelectric point of 8.6 is adsorbed onto the column while the polypeptide NAS is obtained in a non-adsorbed fraction. The non-adsorbed fraction is applied to an anion exchange column by general methods, so that the polypeptide NAS with an isoelectric point of 4.7 is adsorbed onto the column. Thus, the NAS is eluted, desalted and dried.

The polypeptide NAS may also be obtained by genetic engineering methods based on the DNA shown below in (4).

The polypeptide shown above in (A) or (B) can be produced by appropriate synthetic methods, for example solid phase synthetic method, partial solid phase synthetic method and solution synthetic method as well as chemical synthetic methods such as fluorenylmethyloxycarbonyl method (Fmoc method), and t-butyloxycarbonyl method (tBOC method). Additionally, the polypeptide shown in (B) may be obtained by a site-directed mutagenesis including altering the amino acid sequence shown in SEQ ID NO.2 in the sequence listing into an amino acid sequence such that one or several, preferably one to 5 amino acids may be substituted, deleted, inserted, added or inverted.

[0022] (3) Polypeptide PNAS of the invention

The polypeptide PNAS of the invention is a polypeptide shown below in (A) or (B).

(A) A polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing;

(B) A polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing, which polypeptide grows to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

[0023] Herein, (A) a polypeptide comprising an amino acid

sequence shown in SEQ ID NO.3 in the sequence listing is first identified by the inventors as a polypeptide NAS precursor containing the signal peptide and extension peptide of the polypeptide NAS described above in (2). Specifically, (A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing is generated as a precursor of the polypeptide comprising an amino acid sequence shown in SEQ ID NO. 2 in the sequence listing in the cells of the plant and is then processed into (A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing.

Thus, the polypeptide PNAS as an NAS precursor may be (A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing but may be a polypeptide substantially identical to the polypeptide (A), namely (B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several, or preferably 1 to 5, amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing and growing to the mature polypeptide NAS via processing and thereafter can form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS.

The polypeptide NBS is as described above in (2).

[0024] The polypeptide PCAS is preferably glycosylated with a sugar chain, particularly preferably an N-linked sugar chain, because the polypeptide NAS obtained through the cleavage of

the signal peptide (the part comprising the nucleotides 1 to 22 in the amino acid sequence shown in SEQ ID NO. 3) and the extension peptide (the part comprising the nucleotides 136 to 158 in the amino acid sequence shown in SEQ ID NO. 3) can get an increased binding to the polypeptide PBS via processing so that the polypeptide NAS can form neoculin with a higher stability. Among the N-linked sugar chains, an N-linked sugar chain comprising mannose/N-acetylglucosamine/fucose/xylose at a ratio of 3/2/1/1 is preferable. Specifically, the N-linked sugar chain is a sugar chain in the structure shown in Fig. 8. Furthermore, a part of the structure shown in Fig. 8 may have addition, deletion, substitution or modification.

From the standpoint of the binding feature of such N-linked sugar chain, the binding site of the N-linked sugar chain in the polypeptide PNAS may possibly be asparagine-103 in the amino acid sequence shown in SEQ ID NO. 3 in the sequence listing.

[0025] The polypeptide PNAS as described above is a precursor of the subunit NAS forming neoculin and can therefore be obtained by genetic engineering methods based on the DNA of the gene encoding PNAS as shown below in (4). The polypeptide shown in above (A) or (B) can be produced by appropriate synthetic methods, for example solid phase synthetic method, partial solid phase synthetic method and solution synthetic method as well as chemical synthetic methods such as

fluorenylmethyloxycarbonyl method (Fmoc method), and t-butyloxycarbonyl method (tBOC method). Additionally, the polypeptide shown in (B) may be obtained by a site-directed mutagenesis including altering the amino acid sequence shown in SEQ ID NO.2 in the sequence listing into an amino acid sequence such that the amino acid sequence may include the substitution, deletion, insertion, addition or inversion of one or several, preferably one to 5 amino acids.

[0026] (4) DNAs of the invention

The DNAs of the invention are the DNA of the gene encoding the polypeptide NAS described above in (2) and the DNA of the gene encoding the polypeptide PNAS described above in (3).

[0027] Specifically, first, the DNA of the gene encoding the polypeptide NAS is the DNA of the gene encoding the polypeptide NAS described above in (2), more specifically the polypeptide NAS described below in (A) or (B):

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing;

(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

[0028] The DNA of the gene encoding the polypeptide NAS may

be obtained as (A) DNA containing a nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing, and may be obtained as DNA hybridizing with the DNA of a nucleotide sequence substantially identical to the aforementioned nucleotide sequence, namely (B) the DNA of the nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or a nucleotide sequence capable of functioning as probe prepared from at least a part of the aforementioned nucleotide sequence, under stringent conditions and encoding a polypeptide capable of forming the neoculin dimer having a taste-modifying activity together with the polypeptide NBS. Herein, the polypeptide NBS is as described above in (2).

Herein, the phrase "stringent conditions" means conditions under which so-called specific hybrid is formed but non-specific hybrid is not formed. It is difficult to numerically express the conditions clearly. Nonetheless, one example thereof is a condition under which DNAs with high homology, for example 90% or more homology, is hybridized together, while DNAs with lower homology is never hybridized, or a rinse condition for general hybridization, for example a rinse condition of 0.1 × ssc at a salt concentration corresponding to 0.1 % SDS and 65°C.

[0029] Such DNA of the gene encoding the polypeptide NAS can

be obtained for example by extracting mRNA from a fruit of Curculigo latifolia several weeks after pollination, synthetically preparing cDNA with reverse transcription · polymerase chain reaction (RT-PCR), and packaging the cDNA in a phage vector. Then, infection with the phage vector is carried out to obtain a cDNA library. Subsequently, a probe prepared on the basis of the amino acid sequence of the polypeptide NAS clarified in the invention is allowed to identify the intended DNA with a plaque hybridization, and the intended DNA is recovered.

The DNA may also be obtained by PCR using, as a primer, an oligonucleotide synthetically prepared on the basis of the nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence described as SEQ ID NO.1 in the sequence listing. Otherwise, the DNA shown above in (A) or (B) may be synthetically prepared with various commercially available DNA synthesizers.

Additionally, the DNA shown in (B) may be obtained, for example, by site-directed mutagenesis including appropriately introducing mutations such as substitution, deletion, insertion or addition into the nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing. The DNA may also be obtained by known mutation processes.

[0030] Secondly, the DNA of the gene encoding the polypeptide

PNAS is the DNA of the gene encoding the polypeptide PNAS described above in (3), more specifically the polypeptide PNAS shown below in (A) or (B).

(A) The polypeptide comprising an amino acid sequence shown in SEQ ID NO.3 in the sequence listing.

(B) The polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.3 in the sequence listing, which polypeptide grows to the mature polypeptide NAS via processing so as to be able to form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS shown above in (a) or (b).

[0031] The DNA of the gene encoding such polypeptide PNAS may specifically be obtained as (A) DNA containing a nucleotide sequence comprising the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO. 1 in the sequence listing or as (B) DNA hybridizing with the DNA of a nucleotide sequence substantially identical to the aforementioned nucleotide sequence, namely the nucleotide sequence comprising the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or a nucleotide sequence capable of functioning as probe prepared from at least a part of the aforementioned nucleotide sequence, under stringent conditions and encoding a polypeptide growing to the mature polypeptide NAS via processing so as to be able to form the

neoculin dimer having a taste-modifying activity together with the polypeptide NBS. As described in the above (2) is about the polypeptide NBS.

The phrase "stringent conditions" means the same as described for the DNA of the gene encoding the polypeptide NAS.

[0032] Such DNA of the gene encoding the polypeptide PNAS can be obtained, for example, in the same manner as in the case of the mature polypeptide NAS from a fruit of Curculigo latifolia several weeks after pollination. Additionally, the DNA shown above in (A) or (B) may also be obtained by PCR using, as a primer, an oligonucleotide synthetically prepared on the basis of the nucleotide sequence of the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing or may be synthesized with various commercially available DNA synthesizers.

Additionally, the DNA shown in (B) may also be obtained by site-directed mutagenesis including appropriately introducing mutations such as substitution, deletion, insertion or addition into the nucleotide sequence comprising the nucleotides 4 to 477 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing. Additionally, the DNA may be obtained by known mutation processes.

[0033] It is needless to say that the two DNAs of the invention may satisfactorily contain a regulatory element for the nucleotide sequences and a structural genes.

[0034] (5) Taste-modifying composition of the invention

The taste-modifying composition of the invention characteristically contains a dimeric protein neoculin having a taste-modifying activity. Neoculin is as described above in (1).

The taste-modifying composition of the invention may be incorporated as it is but may be added at an appropriate amount to foods or drinks including vegetable juice, fruit juices of for example grapefruit, and various seasoning liquids for cooking for sushi neta (sushi topping), or pharmaceutical agents or the like. The blended amount of neoculin in this case is for example 5 to 5,000 μg/ml, particularly preferably 50 to 500 μg/ml, when the composition containing a neoculin powder highly purified is added to a drink.

Additionally, the taste-modifying composition of the invention may be used after processing into the form of powder, solution, sheet, spray, granule or emulsion, depending on the property of a food or a drink or a pharmaceutical agent as a blend subject.

Examples are now given below.

[Example 1]

[0035] A fruit of Curculigo latifolia was purified by the following procedures to obtain the novel protein with the taste-modifying activity.

[0036] (1) Preparation of crude extract solution

40 liters of pure water were added to about 1 kg of the freeze-dried fruit of Curculigo latifolia (freeze-dried fruit powder in Table 1) for homogenization for 15 minutes. Then, centrifugation at 6,000 rpm for 20 minutes was done to discard the supernatant (the supernatant had no taste-modifying activity). The procedure described above was repeated twice, to obtain the residual precipitate.

Then, 20 liters of 0.05N sulfuric acid were added to the residual precipitate, for homogenization for 10 minutes. Then, centrifugation at 6,000 rpm for 20 minutes was done to recover the supernatant. The procedure described above was repeated twice. The resulting precipitate had no taste-modifying activity.

Then, 2 liters of 1N sodium hydroxide were added to the extract solution for neutralization, to obtain a crude extract solution (0.05N sulfate extract solution in Table 1) containing the active substance.

[0037] (2) Purification on Amberlite IRC-50 column

About 40 liters of the crude extract solution obtained in (1) were passed through an Amberlite IRC-50 column (manufactured by Organo; an 8 cm diameter × 30 cm) equilibrated with 50 mM phosphate buffer, pH 5.5 for adsorption. Continuously, the column was washed with one liter of 50 mM phosphate buffer, pH 5.5, and eluted with 1.5 liters of 50 mM phosphate buffer, pH 5.5 containing 1M sodium chloride, to

obtain a fraction with a taste-modifying activity. Ammonium sulfate was added to the active fraction to 60% saturation, to separate out the active substance, which was centrifuged at 6,000 rpm for 30 minutes. The resulting precipitate was dissolved in 100 ml of 0.2N acetic acid, to recover a solution of the active substance (Amberlite IRC-50 Chromatography in Table 1).

[0038] (3) Purification on Sephadex G-25 column

100 ml of the solution of the active substance as obtained in (2) was applied to a Sephadex G-25 column (manufactured by Amersham Biosciences; an 8 cm diameter × 30 cm) equilibrated with 0.2N acetic acid, for desalting. The solution of the active substance was freeze-dried to obtain a highly purified neoculin powder (Sephadex G-25 Chromatography in Table 1).

The protein content, the activity yield and the purification degree obtained at each purification step are shown in Table 1.

[0039] [Table 1]

	Protein content (g)	Activity yield (%)	Purification degree (-fold)
Freeze-dried fruit powder	1000	100	1
0.05N sulfate extract solution	18	80	45
Amberblite IRC-50 Chromatography	3	55	185
Sephadex G-25 Chromatography	1	36	432

[0040] (4) Verification of purification results

2.5 μg of the purified neoculin powder obtained above

in (1) to (3) was subjected to SDS-PAGE under reducing conditions or non-reducing conditions at a gel concentration of 15 % and subsequently to CBB staining. As shown in Fig. 1, a single band was observed at a position of 20 kDa under non-reducing conditions, while under reducing conditions, two bands were observed at positions of 13 kDa and 11 kDa. This indicates that the resulting neoculin was highly purified and that neoculin was a dimer comprising the each subunit with 13 kDa and 11 kDa. Thus, the individual subunits were designated as neoculin acidic subunit (NAS) and neoculin basic subunit (NBS).

[0041] (5) Verification of taste-modifying activity

1.1 mg of the purified neoculin powder obtained above in (1) to (3) was subjected to Native-PAGE, using 10 % acrylamide gel containing 6M urea. The resulting band was excised out. A sample extracted from the gel with water was freeze-dried. The freeze-dried sample was suspended in 150 μl of water. 50 μl of the resulting suspension was placed into the oral cavities of two panelists. The panelists verified that the sample had a sweet taste and that the sour taste of 0.02M citric acid the panelists placed in the mouths after spewing out the sample was modified into sweet taste. Thus, it was confirmed that the sample had a taste-modifying activity.

Further, a 1/1000 volume of the recovered sample was

subjected to SDS-PAGE, for staining with silver. A single band was observed at a position of 20 kDa, establishing the confirmation that the substance with the taste-modifying activity per se was neoculin.

[Example 2]

[0042] Neoculin obtained in Example 1 was purified further by the following procedures, to make the analysis of the individual subunits constituting neoculin.

[0043] (1) Purification on HiTrap SP Sepharose Fast Flow column

100 mg of the neoculin powder obtained in Example 1 was dissolved in 20 ml of buffer A (50 mM Tris-HCl buffer, pH 7.5 containing 8M urea and 30 mM DTT). Then, the whole volume was applied to HiTrap SP Sepharose Fast Flow column (manufactured by Amersham Biosciences; a 1.6 cm diameter × 2.5 cm) equilibrated with the buffer A. Continuously, the column was washed with 50 ml of the buffer A. Continuously, elution was done with 50 ml of the buffer A containing 1M NaCl, to obtain a purified NBS fraction. Furthermore, 70 ml of the wash fraction was dialyzed against ion exchange water to a volume of 100 ml.

[0044] (2) Second purification on HiTrap SP Sepharose Fast Flow column

To 100 mg of the wash fraction obtained above in (1) was added 8 M urea, 30 mM DTT and 50 mM acetate buffer, pH 4.5 so as to be a total volume of 150 ml (all were expressed as final

concentrations). The resulting mixture solution was applied to HiTrap SP Sepharose Fast Flow column (manufactured by Amersham Biosciences; a 1.6 cm diameter × 2.5 cm) equilibrated with buffer B (50 mM acetate buffer, pH 4.5 containing 8 M urea and 30 mM DTT). Continuously, the column was washed with 50 ml of the buffer B, for elution with 50 ml of buffer B containing 1 M NaCl. Additionally, 200 ml of the wash fraction was dialyzed against ion exchange water to a volume of 250 ml.

[0045] (3) Purification on HiTrap DEAE Sepharose Fast Flow column

To 250 mg of the wash fraction obtained above in (2) was added 8 M urea, 30 mM DTT and 50 mM Tris-HCl buffer, pH 9.0 so as to be a total volume of 350 ml (all were expressed as final concentrations). The resulting mixture solution was applied to HiTrap DEAE Sepharose Fast Flow column (manufactured by Amersham Biosciences; a 1.6 cm diameter × 2.5 cm) equilibrated with buffer C (50 mM Tris-HCl buffer, pH 9.0 containing 8 M urea and 30 mM DTT). Continuously, the column was washed with 50 ml of the buffer C, for elution with 50 ml of buffer C containing 1 M NaCl, to obtain a purified NAS fraction.

[0046] (4) Verification of purification results

The NBS fraction and the NAS fraction obtained above in (1) and (3) were individually dialyzed and freeze-dried, to obtain purified powders. 10 μg each of these purified powders

was subjected to SDS-PAGE. As shown in Fig. 2, a single band was observed in the NAS fraction and the NBS fraction at positions of 13 kDa and 11 kDa, respectively. Thus, it was confirmed that each of the subunits was purified.

[Example 3]

[0047] The amino acid sequence of the NAS fraction constituting neoculin as obtained in Example 2 was analyzed by the following procedures.

[0048] (1) Analysis of N-terminal amino acid sequence

70 μg of the purified NAS powder obtained in Example 2(1) to (3) was subjected to two-dimensional electrophoresis. The gel after electrophoresis was overlaid on a polyvinylidene difluoride (PVDF) membrane, where an electric current passed vertically for transfer. The PVDF membrane after the transfer was stained with SYPRO Ruby protein blot stain (manufactured by Molecular Probes), from which bands were excised out by a general method for the analysis of the N-terminal amino acid sequence with an amino acid sequencer (HP G1005A Protein Sequencing System). In other words, phenylisothiocyanate (PITC) is allowed to react with a free amino residue at the N terminus to prepare a phenylthiocarbamyl derivative (PTC amino acid), which is then released with trifluoroacetic acid in the form of anilinothiazolinone-amino acid. Then, the anilinothiazolinone-amino acid is converted to a stable phenylthiohydantoin (PTH amino acid) in an acidic condition,

for the analysis. In such manner, an amino acid sequence of 40 residues from the N terminus was determined.

[0049] (2) Analysis of inner amino acid sequence

(i) S-Carboxyamide methylation

10 mg of the purified NAS powder obtained in Example 2(1) to (3) was dissolved in 100 ml of 500 mM Tris-HCl buffer, pH 8.0 containing 6 M urea and 20 mM DTT. The resulting solution was left to stand alone at 50°C for one hour. 100 mg of iodoacetoamide was added to and mixed into the solution, which was then shaken in darkness at room temperature for 45 minutes. After the reaction solution was dialyzed and freeze-dried, NAS converted to S-carboxyamide methyl was obtained.

[0050] (ii) Fragmentation with chymotrypsin

S-carboxyamide methylated NAS obtained above in (i) was digested with chymotrypsin in 0.1 M Tris-HCl buffer, pH 8.0 at 37°C for 16 hours. The protein concentration was adjusted to 0.2 mg/ml while the ratio of the enzyme: the substrate was 1:20. The reaction was terminated by a processing at 100°C for 3 minutes.

[0051] (iii) Fragmentation with endoproteinase Asp-N

S-carboxyamide methylated NAS obtained above in (i) was digested with endoproteinase Asp-N in 50 mM phosphate buffer, pH 8.0 containing 0.01 % SDS at 37°C for 16 hours. The protein concentration was adjusted to 1.0 mg/ml while the ratio of the enzyme: the substrate was 1:100. The reaction was terminated

by a processing at 100°C for 3 minutes.

[0052] (iv) Fragmentation with trypsin

S-carboxyamide methylated NAS obtained above in (i) was digested with trypsin in 0.1 M carbonate ammonium buffer, pH 8.5 containing 2 M urea at 37°C for 24 hours. The protein concentration was adjusted to 0.2 mg/ml while the ratio of the enzyme: the substrate was 1:20. The reaction was terminated by a processing at 100°C for 3 minutes.

[0053] (v) Peptide separation and sequence analysis

The chymotrypsin-digested peptide, the endoproteinase Asp-N-digested peptide and the trypsin-digested peptide as obtained by the procedures above in (ii) to (iv) were separated by HPLC using TSKgel ODS-80TsQA column (manufactured by TOSOH; a 4.6 mm diameter × 15 cm). The individual peptides were eluted by an elution method on a linear gradient of acetonitrile containing 0.05 % trifluoroacetic acid.

Fig. 3 shows the HPLC elution pattern of the chymotrypsin-digested peptide as detected at absorbance at 220 nm; Fig. 4 shows the HPLC elution pattern of the endoproteinase Asp-N-digested peptide as detected in the same manner as described above; and Fig. 5 shows the HPLC elution pattern of the trypsin-digested peptide as detected in the same manner as described above.

The peptides detected at 220 nm absorbance and isolated were dried and subsequently analyzed of the inner amino acid

sequences with an amino acid sequencer (Procise 491cLC Protein Sequencing System or Procise 492HT Protein Sequencing System).

[0054] (3) Analysis of C-terminal amino acid sequence

1 nmol of the S-carboxyamide methylated NAS obtained above in (2)(i) was digested with carboxypeptidase A in 50 mM Tris-HCl buffer, pH 7.5 containing 0.15 % SDS at 25°C. The protein concentration was adjusted to 5 mg/ml, while the ratio of the enzyme: the substrate was 1:40. Immediately after the start of the reaction and 6 hours after the reaction, sampling was done. An equal volume of 10 % trichloroacetic acid was added to the reaction solution to terminate the reaction. The reaction solution was left to stand alone at 0°C for 30 minutes and then centrifuged, to recover the supernatant.

Amino acids released in the supernatant were modified into PTC amino acids, for quantitative analysis by HPLC using TSKgel ODS-80 TsQA column (manufactured by TOSOH; a 4.6 mm diameter × 15 cm). The results are shown in Fig. 6.

As shown in the results in Fig. 6, it was confirmed that the C-terminal sequence of NAS was NLSP/R from the C terminus.

[0055] (4) Determination of primary structure

The amino acid sequence determined by the methods is as shown in SEQ ID NO. 2 in the sequence listing and in Fig. 7. In Fig. 7, herein, N in the line 4 represents an amino acid sequence portion determined from the N terminus, while C1-14 represent amino acid sequences of peptides obtained by the

chymotrypsin digestion; D1-4 represent amino acid sequences of peptides obtained by the endoproteinase Asp-N digestion; and T1-4 represent amino acid sequences of peptides obtained by the trypsin digestion. Additionally, the arrow ← represents an amino acid sequence resulting from the C terminus.

[Example 4]

[0056] The amino acid sequence of the NBS fraction constituting neoculin as obtained in Example 2 was analyzed by the same procedures as in Example 3.

Consequently, the amino acid sequence of NBS was shown in SEQ ID NO.6 in the sequence listing and therefore, the amino acid sequence almost completely corresponds to the amino acid sequence of curculin B as a polypeptide already disclosed in JP-A-Hei 6-189771.

[Example 5]

[0057] The sugar chain of the NAS fraction constituting neoculin as obtained in Example 2 was analyzed by the following procedures.

In other words, the peptide with the sugar chain-glycosylated consensus sequence, namely the chymotrypsin-digested peptide C1 of the purified NAS as obtained in Example 3(2) (ii) was recovered, to analyze the sugar composition of the sugar chain using an ABEE sugar composition analysis kit plus S (manufactured by Honen

Corporation).

Specifically, the peptide was treated for the release of sialic acid; then, the sugar was converted to a reduced sugar, which was continuously hydrolyzed with an acid, to cleave all the glycoside bonds contained in the sugar chain of the glycoprotein, whereby releasing the sugars in the forms of monosaccharides. After the generated monosaccharides were labeled and then separated by HPLC using TSKgel ODS-80TsQA column (manufactured by TOSOH; a 4.6 mm diameter × 7.5 cm), detection was performed by 305 nm absorbance for analysis.

As the results of the analysis, the sugar composition of the sugar chain added to NAS was mannose/N-acetylglucosamine/fucose/xylose at a ratio of about 3/2/1/1. Fig. 8 shows the results and a sugar chain structure glycosylated NAS as speculated with reference to sugar chain structures observed generally in plants.

[Example 6]

[0058] The gene encoding NAS constituting neoculin was cloned by the following procedures.

[0059] (1) Preparation of cDNA library

As a material, a fruit of Curculigo latifolia (supplied from the Yamashina Plant Data Institute, Nippon Shinyaku) was used. About 20.6 g of the fruit aged 4 weeks to 8 weeks after pollination was frozen in liquid nitrogen, which was then ground while avoiding thawing. Using 20.6 g of the powder

obtained in such manner as a sample, Poly(A)+mRNA was extracted by mRNA Purification Kit (manufactured by Amersham Bioscience).

From about 4.5 μg of the extracted mRNA, a cDNA library was prepared using cDNA Synthesis Kit (manufactured by Amersham Bioscience). cDNA was inserted via EcoRI adaptor conjugation into λZAPII vector (manufactured by Stratagene). The vector was packaged in a phage using Gigapack III Gold Packaging Extract (manufactured by Stratagene), which was allowed to infect Escherichia coli XL1-Blue MRF'. Thus, a library of about 1.2×10^5 plaques was prepared.

[0060] (2) Preparation of probe

Using 20 mg of the freeze-dried fruit of Curculigo latifolia as a material, the genome DNA was extracted, using DNeasy Plant Mini Kit (manufactured by QIAGEN). Based on the amino acid sequence of NBS as disclosed in JP-A-Hei 6-189771, the NC1S primer shown in SEQ ID NO. 4 in the sequence listing, and the NC1A primer shown in SEQ ID NO.5 in the sequence listing were synthetically prepared. Using the extracted genome DNA as template, PCR was done (one cycle of 94°C for 3 minutes, 42°C for 3 minutes, and 72°C for 3 minutes and 50 cycles of 94°C for 30 seconds, 42°C for 30 seconds and 72°C for one minute). A 469bp DNA fragment encoding a part of NAS was obtained. The DNA fragment was used as probe.

[0061] (3) Plaque hybridization

2×10^4 plaques in the library obtained in (1) were transferred onto a nylon membrane to immobilize DNA. Using subsequently the probe prepared in (2), hybridization was done at 65°C. Using 0.1 × SSC and 0.1 % SDS, rinsing was done at 65°C. Consequently, about 100 plaques were hybridized with the probe. 25 plaques with more intense signals were subjected to secondary screening, for separation into single plaque.

[0062] (4) Determination of nucleotide sequence

The single plaque phage obtained in (3) was co-infected with a helper phage and XL1-Blue MRF', for in vivo excision. Then, a pBluescriptII SK(-) containing the insert cDNA was excised out from the λZAPII vector. The nucleotide sequence of cDNA was determined by dideoxy method.

Consequently, the nucleotide sequence thus determined was as shown in SEQ ID NO.1 in the sequence listing. The amino acid sequence of a polypeptide encoded by a nucleotide sequence comprising the nucleotides 70 to 408 in the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing coincided with the amino acid sequence (the amino acid sequence shown in SEQ ID NO.3 in the sequence listing) of the NAS as obtained in Example 4.

Additionally, an open reading frame (ORF) including the amino acid sequence of CAS was found in a part of the nucleotide sequence shown in SEQ ID NO.1 in the sequence listing, which part comprises the nucleotides 4 to 477. It was verified that

CAS was generated as a precursor peptide (PCAS) containing a signal peptide and an extension peptide. The amino acid sequence of PCAS encoded by the nucleotide sequence comprising the nucleotides 4 to 477 therein is shown in SEQ ID NO.3 in the sequence listing.

[Example 7]

[0063] A vegetable juice was prepared by the following procedures, where neoculin was to be added. The taste-modifying activity was evaluated.

300 ml of water was added to finely cut fresh spinach, green pepper, celery of 135 g, 65 g and 65 g, respectively and 25 g of lemon juice. The resulting mixture was processed in a blender for 5 minutes and then filtered through a nylon mesh with pore size of 0.1 mm, to obtain about 400 ml of a vegetable juice. To 100 ml of the resulting vegetable juice was added 50 mg of the purified neoculin powder obtained in Example 1. The resulting juice was designated as high concentration-added vegetable juice. 10 mg of the purified neoculin powder was added to 100 ml of the vegetable juice, which was designated as low concentration-added vegetable juice. The vegetable juice without any addition was designated as control sample. Four panelists made an organoleptic evaluation. While the bitterness, astringency and sweetness of the vegetable juice without any addition were ranked as score 0 as evaluation score, great enhancement of the individual tastes was marked with

score 2; simple enhancement thereof was marked with score 1; great reduction thereof was marked with score -2; and simple reduction thereof was marked with score -1. The mean of the evaluation results is shown in Table 2.

[0064] [Table 2]

	Bitterness	Astringency	Sweetness
Vegetable juice without any addition	0	0	0
High concentration-added vegetable juice	-1.75	-1.25	1.5
Low concentration-added vegetable juice	-1.0	-0.75	1.0

[0065] From the results in Table 2, it was verified that the neoculin of the invention reduced bitterness and astringency greatly and gave sweetness, so that the neoculin had an effect of enhancing the taste of foods.

[Example 8]

[0066] A grapefruit juice was prepared by the following procedures, where neoculin was to be added. The taste-modifying activity was evaluated.

To 100 ml of a grapefruit juice was added 10 mg of the purified neoculin powder obtained in Example 1, which was defined as a high concentration-added sample. A sample prepared by adding 5 mg of the neoculin powder was defined as a low concentration-added sample, while the grapefruit juice without any addition was defined as control sample. Four panelists made an organoleptic evaluation. While the bitterness, astringency and sweetness of the vegetable juice without any addition were ranked as score 0 as evaluation score, great enhancement of the individual tastes was marked with

score 2; simple enhancement thereof was marked with score 1; great reduction thereof was marked with score -2; and simple reduction thereof was marked with score -1. The mean of the evaluation results is shown in Table 3.

[0067] [Table 3]

	Bitterness	Astringency	Sweetness
Sample without any addition	0	0	0
High concentration-added sample	-1.75	-1.25	1.5
Low concentration-added sample	-1.0	-0.5	0.75

[0068] From the results in Table 3, it was verified that the neoculin of the invention greatly reduced bitterness and suppresses sourness and gave sweetness, so that the neoculin had an effect of enhancing the taste of foods.

[Example 9]

[0069] A seasoning liquid for sushi neta (sushi topping) was prepared by the following procedures, where neoculin was to be added. The taste-modifying activity thereof was evaluated. The seasoning liquid for sushi neta as referred to herein is a seasoning for seasoning sushi neta, by preliminarily coating raw sushi neta or once heat-treated sushi neta or immersing sushi neta therein.

The composition of the seasoning liquid for sushi neta was as shown in Table 4. Further, the seasoning liquid for sushi neta was diluted two-fold for use in this Example, because seasoning liquids for sushi neta are appropriately diluted with water, depending on the kind and amount of sushi neta in use.

[0070] [Table 4]

Raw materials	Blended amount (% by weight)
Brewed vinegar	18
Hydrogenated saccharified-starch syrup	13
pH adjuster	8
Seasoning (amino acid)	4
Edible salt	4
water	53

[0071] To 100 ml of the diluted seasoning liquid for sushi neta was added 11.3 mg of the purified neoculin powder obtained in Example 1, to obtain a neoculin-added seasoning liquid. Shrimp was immersed in the neoculin-added seasoning liquid for 20 minutes, to obtain sushi neta of shrimp (neoculin-added sample). Alternatively, shrimp was immersed in the diluted seasoning for sushi neta in the same manner but without any addition of the neoculin powder to obtain sushi neta of shrimp (control sample). Four panelists ate each one of the neoculin-added sample and the control sample, to evaluate the intensity of the astringency of each of the samples. Consequently, it was confirmed that astringency was highly suppressed in the neoculin-added sample, compared with the control sample.

[Example 10]

[0072] The taste-modifying activity of neoculin and the taste-modifying activity of curculin were compared together by the following procedures.

[0073] (1) Preparation of curculin

Curculin B was expressed by the method disclosed in Examples 1 through 12 in JP-A-Hei 6-189771.

Subsequently, a transformant Escherichia coli expressing curculin B was disrupted with an ultrasonic generator to prepare a suspension, which was centrifuged four times using 25 mM phosphate buffer, pH 6.8 containing 50 mM sodium chloride, for washing. The resulting suspension was dissolved in 500 mM Tris-HCl, pH 9.5 containing 8 M urea and 10 mM DTT, for 2.5-hour reduction at 37°C. To the solution was added a 10-fold volume of 500 mM Tris-HCl, pH 8.5 containing 8 M urea and 0.11 M glutathione in the oxidized form. The resulting mixture was left to stand at room temperature for 3 hours, to glutathionylate the protein. To the glutathionylated protein was added a 10-fold volume of 50 mM Tris-HCl buffer, pH 9.0 containing 4 mM cysteine, for reaction at 4°C for 2 days and subsequent dialysis against 50 mM phosphate buffer, pH 6.8 containing 0.1 M sodium chloride, to form a homodimer. Subsequently, the homodimer was freeze-dried, to obtain a curculin powder as a homodimer of curculin B.

[0074] It was verified by SDS-PAGE that curculin B formed a homodimer. Specifically, 10 μg of the curculin powder was subjected to SDS-PAGE at a gel concentration of 15 % and subsequently to CBB staining, so that as shown in Fig. 9, a single band was observed under non-reducing conditions at a position of about 20 kDa, while it was confirmed that one band was observed at a position of about 11 kDa under reducing conditions. Using the homodimer curculin, the following

organoleptic test was done.

[0075] (2) Comparison of taste-modifying activity

The purified neoculin obtained in Example 1 was dissolved in water to the individual concentrations of 30, 50, 75 and 100 μg/ml, while curculin obtained in (1) was dissolved in water to 100 μg/ml. Using the sweetness felt when 0.1 v/v % acetic acid was then placed in the mouth after 500 μl of the 100 μg/ml curculin solution was once placed in the mouth and then discharged therefrom as a basis, the sweetness felt when 0.1 v/v % acetic acid was then placed in the mouth after 500 μl of a neoculin solution at each of the concentrations was once placed in the mouth and then discharged therefrom was evaluated by 3 panelists. Compared with the sweetness using the curculin solution, the sweetness at the same level was marked with score 0; slightly stronger sweetness was marked with score 1; stronger sweetness was marked with score 2; far stronger sweetness was marked with score 3; slightly weaker sweetness was defined as -1; weaker sweetness was marked with score -2; far weaker sweetness was defined as -3. The mean of the evaluation results is shown in Table 5.

[0076] [Table 5]

	Neoculin concentration (μg/ml)			
	30	50	75	100
Evaluation score	1.33	2.33	2.5	3.0

[0077] The results in Table 5 show that the taste-modifying activity of neoculin was far stronger than the taste-modifying

activity of curculin.

[Industrial applicability]

[0078] In accordance with the invention, a novel dimer protein neoculin with an excellent taste-modifying activity is provided, which is in a heterodimer structure unlike curculin. Using the protein, a novel taste-modifying composition practically applicable to foods and the like can be provided.

In accordance with the invention, additionally, the amino acid sequences of the subunits constituting the protein are provided. By an appropriate synthetic method following the amino acid sequences, the protein can be provided.

In accordance with the invention, still further, the DNA of the gene encoding the protein is provided, which enables to provide the protein by selecting an appropriate host and with a use of genetic engineering technique.

[Brief description of drawings]

[0079] [Fig. 1] shows a pattern obtained by subjecting purified neoculin powder to SDS-PAGE under reducing or non-reducing conditions and then staining the resulting products with CBB.

[Fig. 2] shows a pattern obtained by subjecting purified powders of NAS and NBS to SDS-PAGE and then staining the resulting products with CBB.

[Fig. 3] is an HPLC separation of peptides obtained with chymotrypsin-digestion from S-carboxyamide methylated NAS.

[Fig. 4] is an HPLC separation of peptides obtained via

digestion with endoproteinase Asp-N from S-carboxyamide methylated NAS.

[Fig. 5] shows a chart of the quantitative analysis of free amino acids via trypsin digestion of S-carboxyamide methylated NAS.

[Fig. 6] shows the quantitative analysis of free amino acids from S-carboxyamide methylated NAS (1 nmol) via digestion with carboxypeptidase A.

[Fig. 7] depicts the amino acid sequence of NAS.

[Fig. 8] depicts a sugar chain structure speculated from the analysis of the sugar composition of the sugar chain of NAS.

[Fig. 9] shows a pattern obtained by subjecting curculin to SDS-PAGE under reducing or non-reducing conditions and then staining the resulting products with CBB.

[Description of symbols]

[0080] In Fig. 7, N in the line 4 shows an amino acid sequence part determined from the N terminus; C1-14 show amino acid sequences of peptides obtained via chymotrypsin digestion; D1-4 show amino acid sequences of peptides obtained via digestion with endoproteinase Asp-N; and T1-4 show amino acid sequences of peptides obtained via trypsin digestion. Additionally, ← shows an amino acid sequence determined from the C terminus.

[Sequence Listing]

SEQUENCE LISTING

<110> Mitsukan Group Corporation

<120> New taste modifying protein and DNA encoding same, and application of the same

<130> A-358

<150> JP2004-19251
<151> 2004-01-28

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Gly Ser Gly Cys Arg Leu Thr Leu Leu Ser Asp Gly Asn Leu Val Ile
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Tyr Asp His Asn Asn Asn Asp Val Trp Gly Ser Ala Cys Trp Gly Asp
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Asn Gly Lys Tyr Ala Leu Val Leu Gln Lys Asp Gly Arg Phe Val Ile
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Tyr Gly Pro Val Leu Trp Ser Leu Gly Pro Asn Gly Cys Arg Arg Val
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Asn Gly

[Name of document] Abstract

[Abstract]

[Problem] To find a substance with a better taste-modifying function and determine the structure of the taste-modifying substance, as well as to elucidate the structure thereof at a gene level and determine the primary structure of the substance and obtain the gene encoding the substance. Additionally, it is an object of the invention to provide a novel taste-modifying composition characteristically containing the taste-modifying substance.

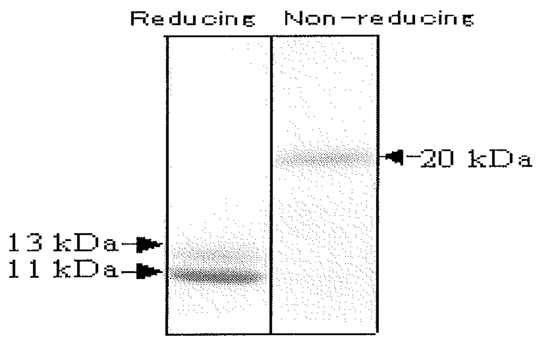
[Means for solution] A polypeptide NAS shown below in (A) or (B), a protein dimer neoculin comprising the polypeptide NAS and the polypeptide NBS and having a taste-modifying activity:

(A) a polypeptide comprising an amino acid sequence shown in SEQ ID NO.2 in the sequence listing;

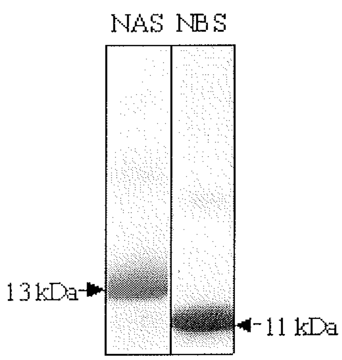
(B) a polypeptide comprising an amino acid sequence with the substitution, deletion, insertion, addition or inversion of one or several amino acids in the amino acid sequence shown in SEQ ID NO.2 in the sequence listing, which polypeptide can form the neoculin dimer having a taste-modifying activity together with the polypeptide NBS.

[Selected drawing] None

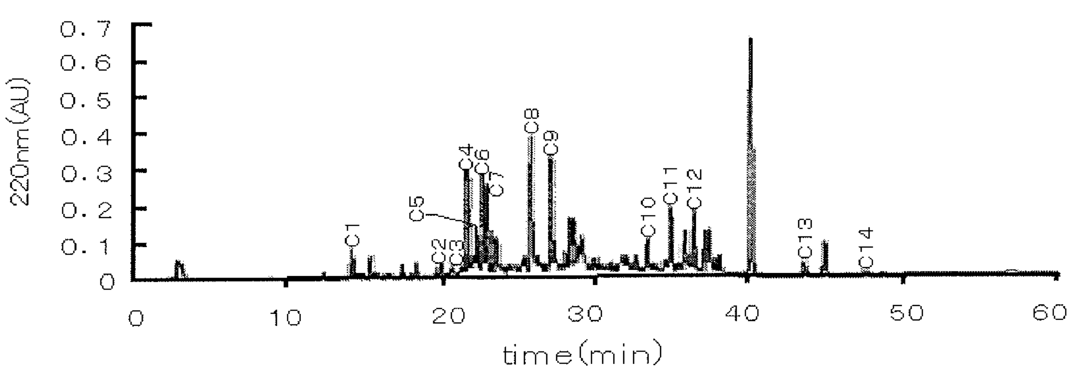
[Name of document] Drawing
[Fig. 1]



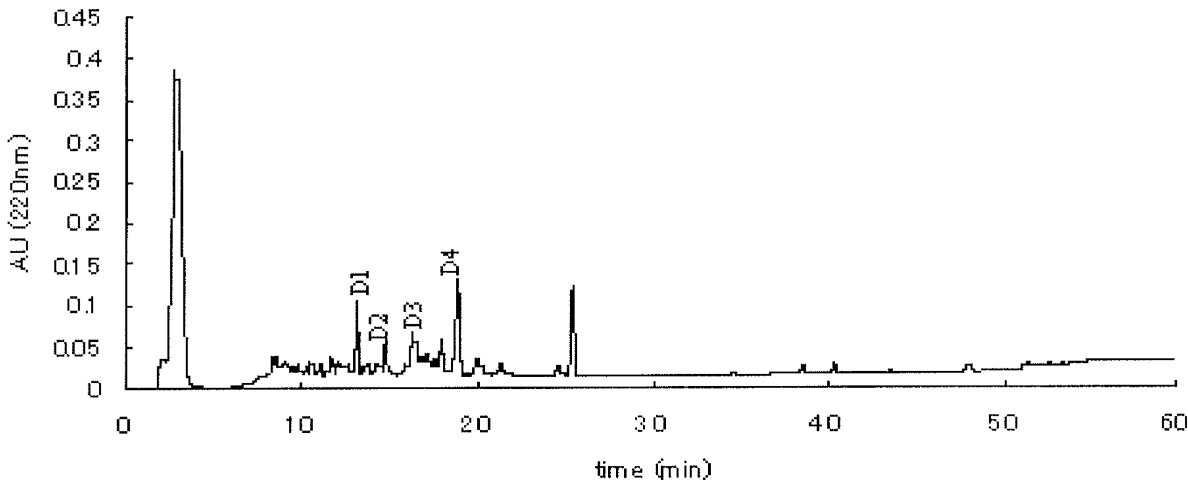
[Fig. 2]



[Fig. 3]



[Fig. 4]



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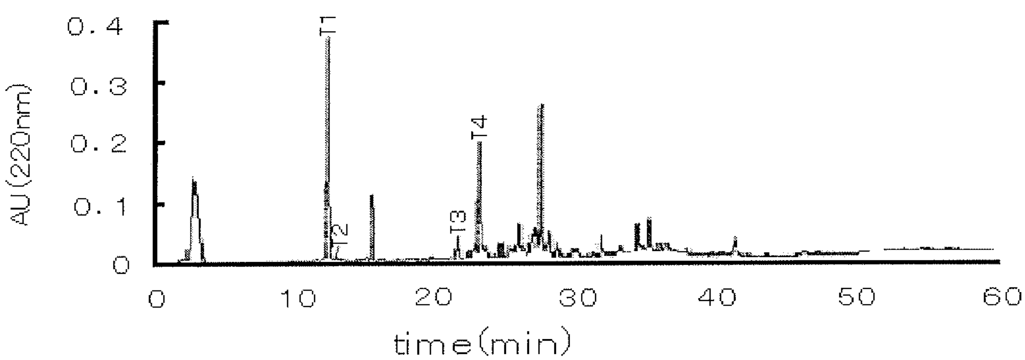

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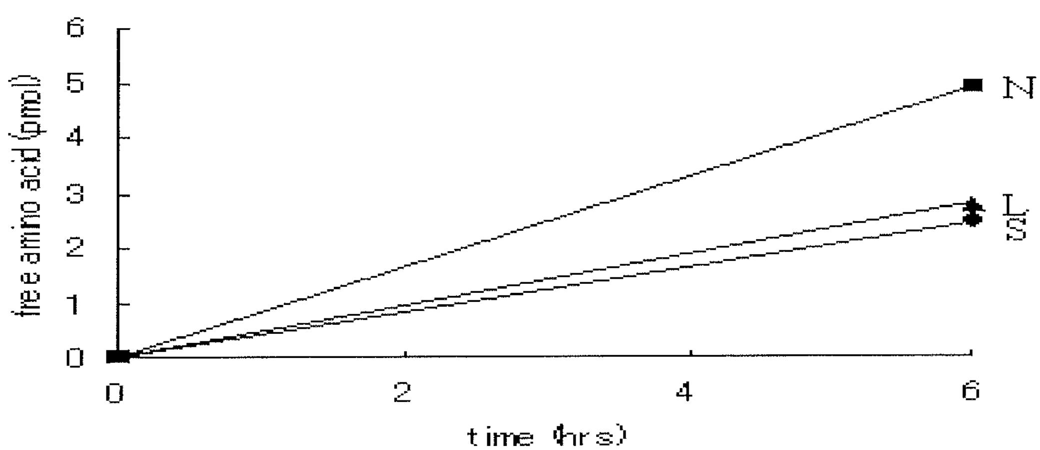
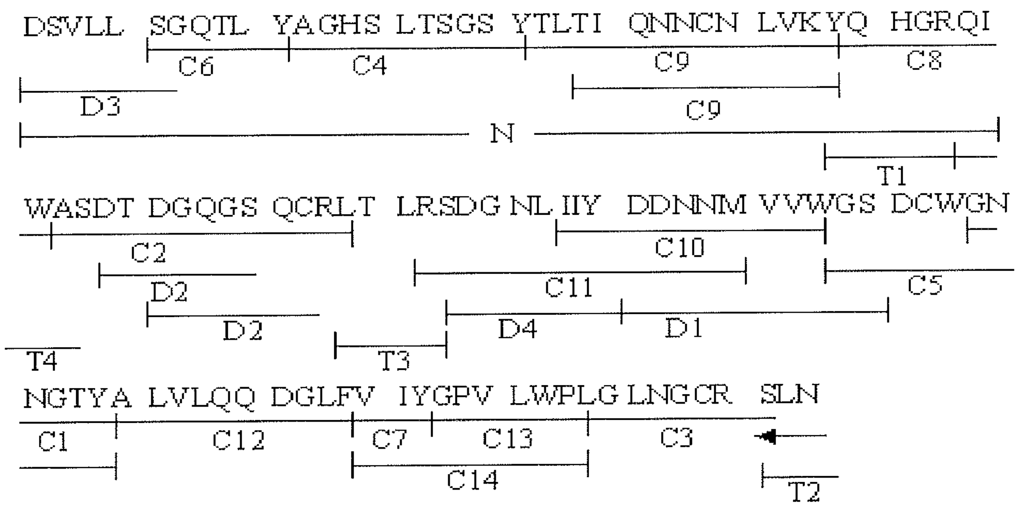
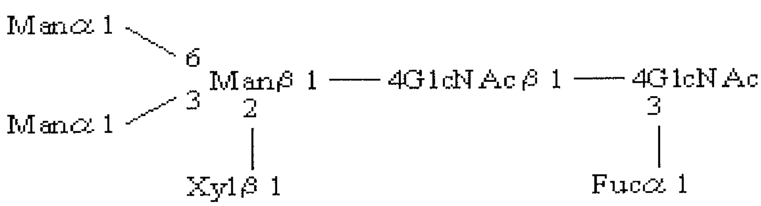


[Fig. 7]



[Fig. 8]



[Fig. 9]